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<b>14. ABSTRACT</b> Traumatic Brain Injury or TBIs are common across both military and civilian populations. In the US, the CDC reports an annual TBI incidence of 1.7 million, with 580,000 TBI-associated deaths in the decade 1997-2007. Low-level laser therapy (LLLT) is unique among the many therapies tested clinically for TBI. Its mechanism of action is biostimulation by near-infrared (NIR) light. In the proposed work, through both clinical and preclinical investigations, we plan to further investigate the safety and utility of LLLT for acute TBI.						
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## Introduction

Traumatic Brain Injury or TBIs are common across both military and civilian populations. In the US, the CDC reports an annual TBI incidence of 1.7 million, with 580,000 TBI-associated deaths in the decade 1997-2007. Worldwide estimates place the number of annual deaths and hospitalizations associated with TBI at 10 million. Low-level laser therapy (LLLT) is unique among the many therapies tested clinically for TBI. Its mechanism of action is biostimulation by near-infrared (NIR) light. In this technical report we have described the progress that has been made on the proposed preclinical and clinical research studies in order to better understand TBI and the potential role of LLLT as a therapy.

## Keywords

Traumatic Brain Injury, TBI, Low level light therapy, LLLT, near-infrared light, NIR

## Overall Project Summary

In the first year of the project we worked toward accomplishing the following objectives:

***Objective 1(i): Acquire and test optical performance of two LLLT device helmets from Photomedex inc.***

The LLLT devices were received in mid-March 2014 and underwent validation testing. A custom test fixture for fluence measurement was constructed to provide validation of the helmet performance and consists of a photodiode power sensor within a black plastic housing unit to confirm the output of each LED matrix in the helmet. We tested each helmet to ensure they perform according to the product specifications. Weekly testing was performed on the LLLT devices through Q3/Q4 and has confirmed the product continues to perform according to the product specifications.

***Objective 1(ii): Conduct double-blinded placebo-controlled study of acute LLLT for TBI including collection of neuroimaging, biochemical, and clinical outcome data.***

Objective 1(ii) was originally scheduled to begin in Q2 of year 1. However, we did not receive the LLLT device from Photomedex until near the end of Q2. In addition, there were minor changes requested following the DoD HRPO review of the MGH approved study protocol. The changes were submitted and approved by HRPO. We received final MGH IRB approval in mid-April. The USUHS IRB approval was also reviewed and approved by the HRPO. In addition to obtaining IRB approvals we have put together the necessary infrastructure for the clinical study.

Screening and recruitment for the study began in June 2014. To date, we have screened over 200 subjects and based on our current protocol only 2 of these subjects were potentially eligible for participation. However, neither of these potential subjects enrolled in the study. Due to the unanticipated low eligibility and enrollment for our study we held an investigator meeting September 25th with our collaborator and TBI expert, Dr. Ramon Diaz-Arrastia from USUHS. We identified 2 study changes that will increase the number of eligible subjects: 1) expand our inclusion criteria from a Glasgow coma scale (GCS) 9-12 to a GCS of 9-15 with 13-15 requiring

an abnormal head CT (this matches the DoD definition of moderate TBI); 2) apply the helmet for 3 days instead of 7 (existing preclinical data of LLLT in mice does not demonstrate a difference between 3 and 7 days of treatment). We are optimistic that with these changes we can increase the number of potential subjects without compromising the scientific integrity of the study.

We will be submitting a protocol amendment with the proposed changes to our IRB immediately.

In addition to the recruitment and screening we have finalized optimizing several of the MRI sequences that will be used in the study protocol for assessing the efficacy of LLLT at baseline and in sub-acute and chronic stages. These sequences, including fMRI and DTI, have been carried out in the Lunder 6 MRI scanner (Siemens MAGNETOM Skyra; Siemens Medical Systems, Erlangen, Germany) located within the MGH Neuro ICU.

***Objective 1(iii): Perform intermediate and end-study analyses of MRI data to assess: 1) white matter integrity difference between the control and placebo groups using DTI metrics, 2) cerebrovascular reactivity difference between the control and placebo groups, and 3) cerebral perfusion using ASL perfusion maps.***

We will perform the analyses later in time once we have collected more clinical data. To date we have nothing to report.

We have also finalized optimizing several of the MRI sequences

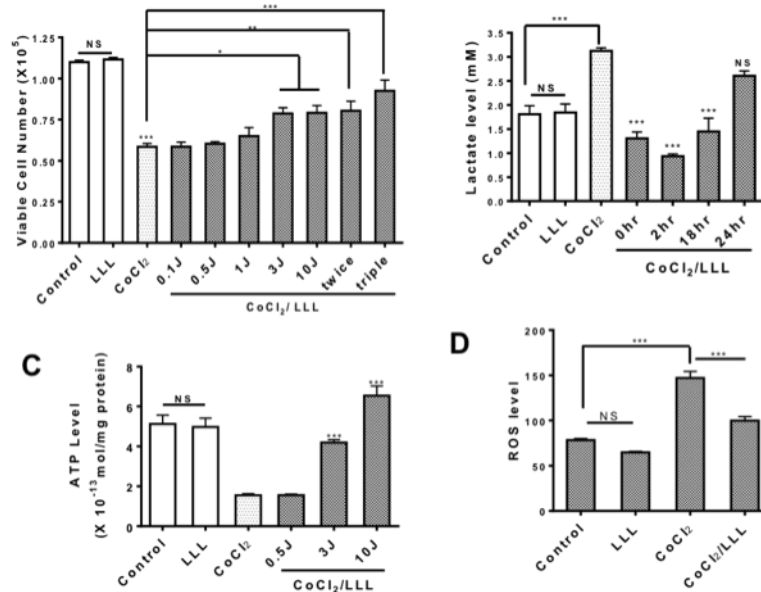
***Objective 1(iv): Perform an end-study analysis of clinical measures to quantify difference in functional improvement associated with LLLT.***

We will perform the end-study analysis once we have collected the clinical data. To date we have nothing to report.

***Objective 2(i): Investigate the effect of LLLT on microglial activation in mouse models of TBI.***

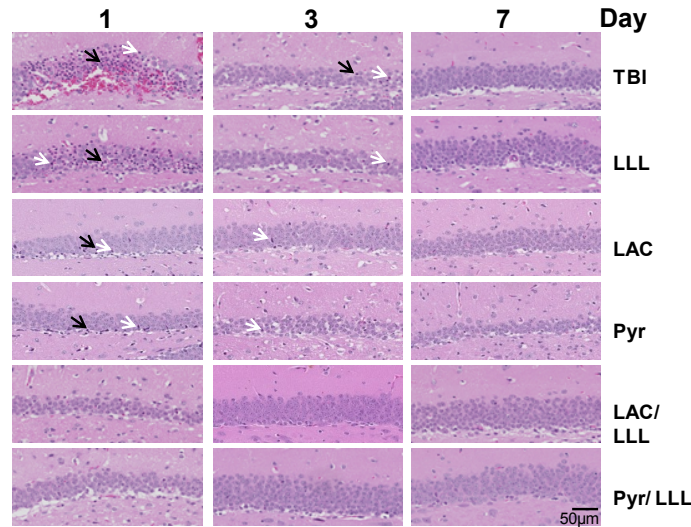
Objective 2(i) was scheduled to begin in Q2 of year 1. During this time we successfully prepared the protocol, submitted to the MGH IACUC and received approval for the study. We have also received DoD review and approval for the preclinical investigation.

We have performed experiments and found that high levels of glycolysis (Fig. 1B), reduced ATP generation (Fig. 1C), and increased formation of reactive oxygen species (ROS) (Fig. 1D), prone to apoptosis (Fig. 1A) in hypoxic neurons could be significantly reversed by low level light therapy (LLLT) in vitro study. The effect of LLLT was furthered by a combination with metabolic substrates like pyruvate or lactate. When the injured brain was treated with LLLT, along with intraperitoneal injection of pyruvate, the mice were partially protected from secondary brain damage in the cortex tissue at the injured site and completely protected the hippocampus tissue from any secondary damage (Figure 2), whereas LLLT or pyruvate treatments alone or untreated control mice showed damage in the hippocampus tissue in single treatment or cortical injury progressed into the hippocampus region in the control mice (Figure 2, day 1). These results suggest a role for metabolic imaging by PET in LLLT, an opportunity that will be explored in more detail in subsequent work.



**Figure 1: LLL retains mitochondrial functions under hypoxia in vitro**

(A) Prevention of hypoxia-induced cell death by LLL. SH-SY5Y cells were treated with 150μM cobalt chloride (CoCl<sub>2</sub>) for 2 hr followed by LLL illumination at indicated doses: twice, treatment with LLL at 3J/cm<sup>2</sup> in 0 and 2 hr post-CoCl<sub>2</sub> incubation and triple, LLL treatment at 0, 2, and 18 hr post-CoCl<sub>2</sub> incubation. (B) Lactate production. The cells were treated with or without CoCl<sub>2</sub> and then illuminated with 3J/cm<sup>2</sup> LLL at indicated times after CoCl<sub>2</sub> administration, and lactate accumulation was measured 48h after CoCl<sub>2</sub> administration. (C) Effects of CoCl<sub>2</sub> and LLL on ATP production. The cells were subject to CoCl<sub>2</sub> treatment, followed by an indicated dose of LLL treatment in 2 hrs. ATP levels were measured 1hr after LLL illumination. ROS production (D) were measured 4 hr after CoCl<sub>2</sub> addition or 2 hr after LLL treatment. All results were expressed as means ± SEM. n=8; \*P<0.05, \*\*P<0.01, \*\*\*P<0.001, and NS, non-significance, respectively.



**Figure 2. Combinational therapy but not single therapy protects the hippocampus of injured mice.** The mice were intraperitoneally administered lactate (Lac) or pyruvate (pyr) 1hr post-TBI, or exposed to LLL 4hr after injury, or treated with both protocols. The region of the hippocampal dentate gyrus was analyzed histologically on a high magnification. The black arrows indicate one of the infiltrated leucocytes, and the white arrows indicate one of the necrotic cells in each field. N=9.

**Methods:** Eight-week-old female C57BL/6 mice were used for all *in vivo* studies. Mice were subjected to closed head TBI by a standard controlled cortical impact on the left lateral with closed skull and scalp. LLLT was performed at 4hr post-TBI using an infrared diode laser of 810nm (Acculaser, PhotoThera Inc, Carlsbad, CA). Briefly, the mouse was positioned on a plate and covered by aluminum sheet with a 1 cm diameter hole to expose the contusion site on the head. The laser's pulse frequency was 10-Hz, pulse duration 50ms, average irradiance

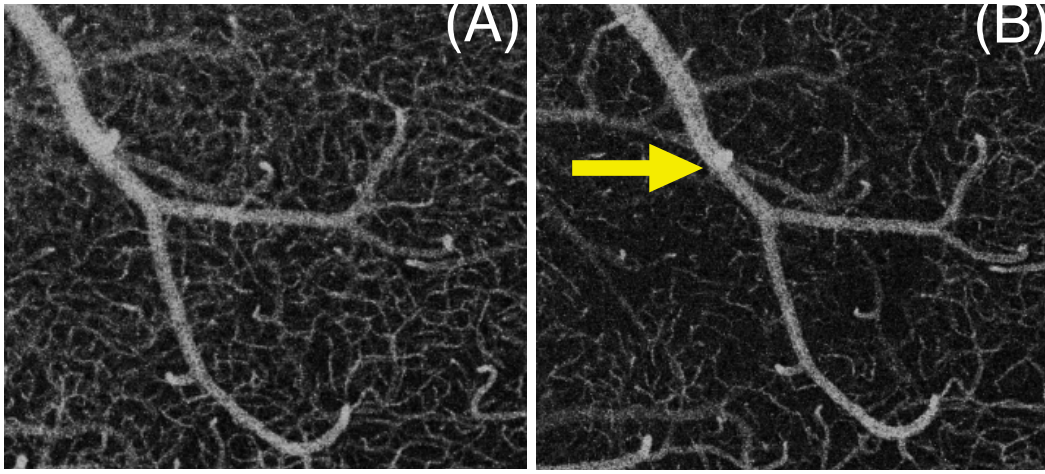
pyruvate was administered intraperitoneally at a dose of 1,000mg/kg at 1 hr post-TBI or 3 hr before the mice were treated with LLLT. The severity of the brain injury was identified by standard histological examination.

***Objective 2(ii): Image cerebrovascular dysfunction with and without LLLT in chronic mouse models of TBI.***

Objective 2(ii) began in Q2 of year 1. During this time we successfully prepared the protocol, submitted to the MGH IACUC and received approval for the study. We have also received DoD review and approval for the preclinical investigation.

The main goal of this objective is to characterize the changes in blood flow in the brain following TBI, and to investigate the effect of LLLT on the altered perfusion. This work is built on the use of an in-house microscopy termed Optical Frequency Domain Angiography (OFDA) which provides detailed visualization of cerebrovascular perfusion in both normal and injured brain sites.

We have developed a new surgical model that allows both injury and OFDA imaging without requiring replacement or removal of an optical window (see Methods). Using this model, we optimized the OFDA system microscope and imaging algorithms to provide highly detailed visualization of the normal brain microvasculature (Figure 1a) and the altered perfusion following injury (Figure 1b). The reduced number of visible vessels in Figure 1b indicates a decrease in perfusion following injury, and specifically a reduction or cessation of flow within capillaries while flow is maintained in the larger arterioles and venules. Blood flow returned to normal within 6 to 8 hours after the initial insult. We are now characterizing in detail the extent and timecourse of TBI-induced microvascular perfusion changes. In Y2, we will investigate if LLLT attenuates the hypoperfusion following injury, or if it accelerates the recovery (an amendment for adding LLLT will be submitted to IACUC/ACURO prior to performing this work). If LLLT is seen to effect microvascular perfusion after TBI, it will indicate both a mechanisms of action and provide a strategy for monitoring the effect of LLLT in clinical settings (for example, using perfusion MRI).



**Fig. 1. (A)** Microphotograph of normal cerebrovascular network in a mouse with a chronic cranial window using OFDA (Optical Frequency Domain Angiography). **(B)** Microphotograph taken ten minutes after mild traumatic brain injury in the same animal. Blood flow stops in small capillaries whereas arterioles and venules, indicated by the yellow arrow, remain perfused.

## ***Materials and Methods***

### ***Polyurethane Cranial Window Model***

We have developed a mouse model for chronic cranial window imaging for mild traumatic brain injury (mTBI). Current chronic rodent models rely on a glass coverslip that is removed right before the injury and replaced afterwards. This could potentially confound experimental results due to inflammation and microbleeding. In our chronic mouse model, the glass coverslip has been replaced by a flexible, biocompatible film. The injury is then delivered directly through this material; therefore avoiding unnecessary manipulation of the window prior to the insult.

To perform a cranial window placements, the mouse was anesthetized using Ketamine/Xylazine (90/9mg/kg). Buprenorphine (0.1mg/kg s.c.) was administered thirty minutes prior to surgery. For this procedure the head of the animal is fixed by a stereotaxic apparatus. The skin on top of the frontal and parietal regions of the skull is cleaned with antimicrobial solution and removed in a rectangle-shape from the base of the skull. Using a high speed air-turbine drill with a burr-tip 0.5 mm in diameter, a groove is made on the margin of the drawn circle. This groove is made thinner by cautious and continuous drilling of the groove until the bone flap becomes loose. Cold saline is applied during the drilling process to avoid thermal injury of the cortical regions. The window is sealed with a 7-mm custom-made stainless-steel ring glued with a thin, clear biocompatible polymer film that replaces the traditional coverslip used in standard cranial windows. This is glued to the bone with histocompatible cyanoacrylate. Buprenorphine (0.1 mg/kg s.c.) was administered every 8-12 hours after the first dose and for up to 72 hours after the procedure. The animal is allowed at least 1 week time to stabilize before further procedures are performed.

### ***Controlled cortical impact injury***

We used a controlled cortical injury (CCI) device similar to those previously described in the literature (Flierl et al., *Nature Protocols* 2009) to produce a mild traumatic brain injury, one that disrupts cerebrovascular perfusion but does not fully destroy the pre-injury vascular network. To administer the CCI injury, mice will be anesthetized with inhalant anesthesia. The head of the



the cranial window remains in place, and the injury is performed through a thin film. Buprenorphine (0.1 mg/kg s.c.) was administered 30 minutes before the procedure, then every 8-12 hours after the first dose and for up to 72 hours after the procedure.

### ***Optical frequency domain angiography (OFDA)***

Optical frequency domain angiography (OFDA) is a non-invasive optical imaging modality. Using low-power infrared light, OFDA permits visualization of tissue microstructure, typically at depths of 1.5-2.5 mm and with spatial resolution elements of 2-20 microns, and allows quantitative blood flow measurements in deeper tissues.

Prior to imaging, the mouse was anesthetized using inhalant anesthesia and placed on a polycarbonate stage equipped with a heating pad to maintain body temperature. The duration of each imaging session is approximately 30 minutes. A heating pad was placed under the animal to maintain the animal's temperature at ~37 C during the imaging session. Effectiveness of anesthesia was monitored by respiration rate, toe and tail pinch, and muscular relaxation. After imaging, the animal was placed in the cage and allowed to recover from anesthesia.

### ***Experimental Design***

First, we performed cranial window surgery in wild-type C57BL/6 mice. Seven days post-procedure, we imaged with OFDA to confirm absence of inflammation and/or microbleeding that could potentially confound our experimental results. This imaging session served as baseline. Later, the mouse underwent a controlled cortical impact. After the injury, we took an image to study the changes in cerebrovascular perfusion.

### ***Objective 2(iii): Quantify neurogenesis in mouse models of TBI with and without LLLT.***

Objective 2(iii) began in Q3 of year 1. During this time we successfully prepared the protocol, submitted to the MGH IACUC and received approval for the study. We have also received DoD review and approval for the preclinical investigation.

We have established experimental setup for conducting the preclinical animal study and will conduct experiments in Q1 of Year 2.

## **Key Research Accomplishments**

The following were accomplished in the 1st year of funding:

- LLLT device helmet was received and tested for performance and safety, and received approval from the MGH Biomedical Engineering Department.
- Constructed test fixture for calibration testing of helmets to confirm consistent optical fluence throughout the study
- Final IRB and DoD approval of clinical study protocol
- Completed MRI sequence programming and hardware modification to allow physiological monitoring during scans (critical for functional sequences)
- Identified and appointed a DSMB and Research Monitor
- Prepared Standard Operating Procedures (SOPs) to ensure study compliance for subject identification and recruitment, consent process and enrollment, device use, clinical assessments, MR imaging, subject follow up, study monitoring, and DSMB meetings to review study data

- Developed case report form for data capture
- Added Emergency Department clinical research assistant to study protocol for recruitment optimization
- Performed study initiation meeting with study staff
- Conducted hands on training for conducting neurocognitive assessments
- Performed extensive screening of over 200 head trauma admissions to MGH, however, no subjects have been enrolled to date due to unanticipated
- Held investigator meeting to identify potential solutions for slower than anticipated enrollment
- IACUC and DoD approval of the 3 planned animal protocols
- Began experimental animal protocols and obtained preliminary results

## **Conclusion**

The need to improve care for TBI in the civilian and military populations is broadly acknowledged. Acute LLLT for TBI has shown promising results in preclinical studies, and the proposed clinical study is designed to perform necessary pilot studies of acute LLLT in human patients. Specifically, the study will establish safety, demonstrate mechanistic activity, obtain pilot data on effect size for clinical outcome measures, and identify imaging and biochemical biomarkers for use in LLLT optimization and dosimetry studies. In addition, TBI remains a consistent public health challenge in civilian populations. If proven effective, acute LLLT would confer a significant benefit to the broad population by reducing chronic deficits associated with TBI. Success in the acute setting would also suggest follow-up clinical studies of LLLT for the large population of TBI patients with chronic disabilities.

## **Publications, Abstracts, and Presentations**

To date we have nothing to report.

## **Inventions, Patents, and Licenses**

To date we have nothing to report.

## **Reportable Outcomes**

To date we have nothing to report.

## **Other Achievements**

To date we have nothing to report.